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In this research, experiments and simulations were performed to determine the effects of capacitive VS conductive coupling for the electroporation of cells. The experiment is performed with a fast risetime pulser to expose samples of cells to electric fields at peak voltage of 24.4 kV, 0.6 ns risetime, and 1.6 ns FWHM. Experiments performed compare the direct-conductive connection of the cell suspension versus a capacitively coupled cell suspension. The main conclusion of this research is that, for the parameters of this experiment, electroporation does not occur for capacitive coupling (Displacement Current). Conduction current is required for electroporation to occur.

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Contact Versus Noncontact Cell Electroporation

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Abstract

In this research, experiments and simulations were performed to determine the effects of capacitive VS conductive coupling for the electroporation of cells. The experiment is performed with a fast risetime pulser to expose samples of cells to electric fields at peak voltage of 24.4 kV, 0.6 ns risetime, and 1.6 ns FWHM. Experiments performed compare the direct-conductive connection of the cell suspension versus a capacitively coupled cell suspension. THE MAIN CONCLUSION OF THIS RESEARCH IS THAT, FOR THE PARAMETERS OF THIS EXPERIMENT, ELECTROPORATION DOES NOT OCCUR FOR CAPACITIVE COUPLING (DISPLACEMENT CURRENT): CONDUCTION CURRENT IS REQUIRED FOR ELECTROPORATION TO OCCUR. The magnitude of the electric field was 15kV/cm in both cases. However, in the case of direct-conductive connection a cell in the center of the tube experienced an electric field in one direction only, whereas a cell in the center of the tube in the capacitive coupling case measures an electric field that reverses direction. From the MAGIC simulation data of the capacitively coupled case we have shown that the integral of the bipolar pulse is zero after the pulse has passed. The direct-conductive connection case is different, in that it is left with a net polarization after the pulse is applied.

Introduction

Cell electroporation with electric fields coupled capacitively or radiated would be a powerful technique both for researchers using electroporation for transfection or clinically for electrochemotheraputic treatment. In this paper the results of experiments and simulations performed to determine the effects of using capacitive coupling for the electroporation of cells are presented. The focus is on reversible cell electroporation although apoptosis is seen in treated cells.

The experiment is performed with a fast risetime pulser to expose samples of cells to electric fields. The cells were exposed to the fields by either a direct conducting connection or by means of capacitive coupling through a test tube into the cell sample. A diagram of the experimental system is shown in Figure 1. The pulser is connected to a pulse compressor/peaking switch, which shortens the pulses and decreases the risetime. The antenna is housed in a sealed plastic box which is filled in SF6 to avoid breakdown across the antenna. The pulser is capable of an output of 33 kV with subnanosecond risetime. The pulse parameters used in these experiments are a peak voltage of 24.4 kV, 0.6 ns risetime, and 1.6 ns FWHM. Pulses are measured using a calibrated probe in the parallel plate antenna system shown in Figure 2. A typical pulse waveform in shown in Figure 3. Experiments performed compare the direct connection of the cell suspension to a capacitively coupled cell suspension.

The particular regime we are exploring has not been published on to our knowledge although others have examined bioeffects of pulses of similar duration and electric field [1]. There have been many studies of the effects of 10s of ns duration pulses with few ns ristimes [2-10] and of subnanosecond pulses at very high fields of up to 1MV/m [11,12].

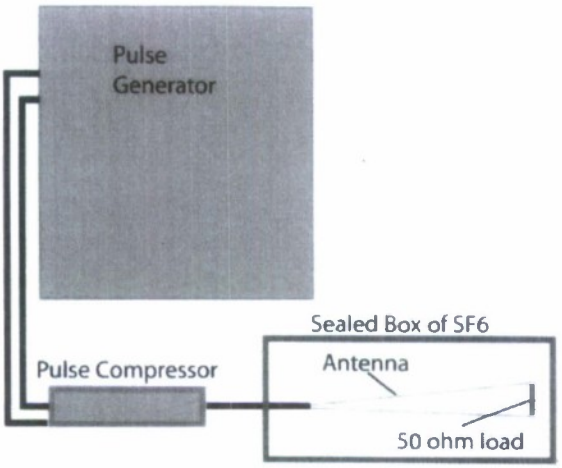

Figure 1. Experiments are performed on the antenna in a sealed box filled with SF6 to prevent arcing.

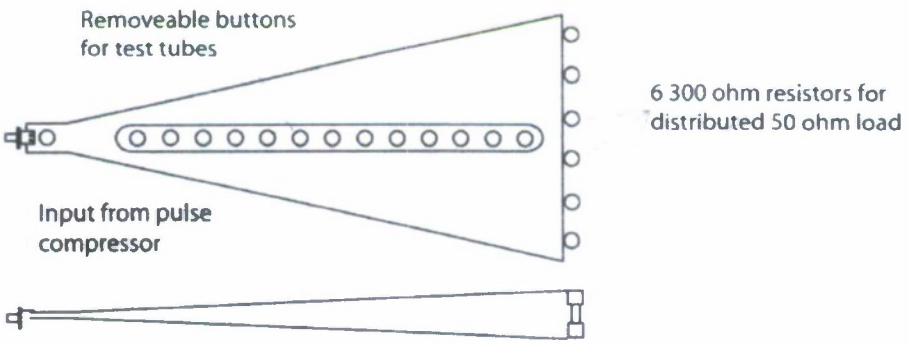

Figure 2. Antenna where the cells are placed as either part of the load or across the plates for capacitive coupling.

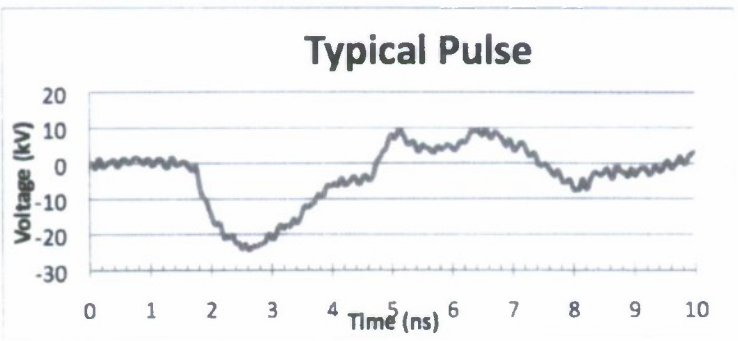

Figure 3. Output from pulse compressor to antenna.

Circuit Model

A simple circuit model of the experiment is used to describe the capacitive coupling of the pulse to the cell suspension. The frequency used in the voltage calculations is taken as that corresponding to the half cycle of 2 ns. This corresponds to about 500 MHz or an angular frequency ω of 3.1×10^9 rad/s. The value of ω is used to determine the magnitude of the impedances. The impedance of the test tube containing the cell suspension is determined from measured parameters and the assumption that the dielectric constant of the suspension is similar to that of water. The contributions from resistive and capacitive impedance are compared in order to determine the conduction current that will flow through the sample. The coupling from the antenna into the cells is purely capacitive. The coupling capacitance is estimated from the formula for a coaxial capacitor

$$C = \frac{2\pi\epsilon_r}{\ln\left[\frac{r_{out}}{r_{in}}\right]}L \tag{1}$$

where L is the length of the coupling region which we take here to be ½ the thickness of the transmission line antenna plates. The numerical result for the 3mm OD 1.8mm ID pyrex tubes with ε_r of 5.1 is 1.7 pF. The resistance of the suspension is given by

$$R = \rho\frac{d}{A} \tag{2}$$

where d is the distance between the plates and A is the area of the suspension. Using the measured conductivity of 1.14 the resistance is 1725 ohms. The capacitance across the suspension is estimated from the parallel plate capacitor formula

$$C = \epsilon_r\frac{A}{d} \tag{3}$$

Assuming the cell suspension has a similar permittivity to water and taking d to be the spacing between the transmission line plates the capacitance is 0.36 pF. The resistance and capacitance of the cell suspension is in parallel and the capacitance from the upper and lower coupling to the transmission line will be in series with the cell suspension. The circuit diagram of this system is shown in Figure 4. An equivalent model of this circuit can be made to simplify the understanding of the voltage drops across the coupling capacitors and compare that to the voltage drop across the cell suspension. There is a voltage drop across the top and bottom capacitors that couple from the transmission line to the cell suspension. The remaining voltage will be across the cells and that voltage contols electroporation. The impedance contribution from the top and bottom capacitors can be combined into a single impedance Z_c. The voltage across the cells can now easily be estimated as the voltage across the divider comprised of the Z_{sol} and Z_c.

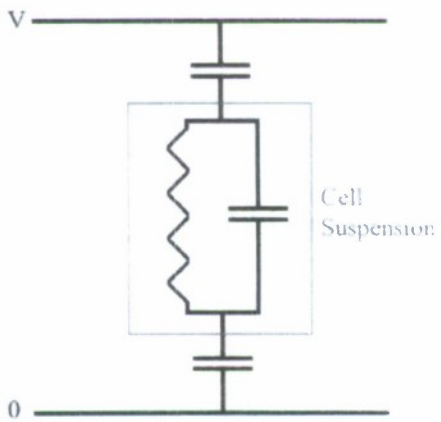

Figure 4. Circuit of cell suspension test tube inserted into transmission line.

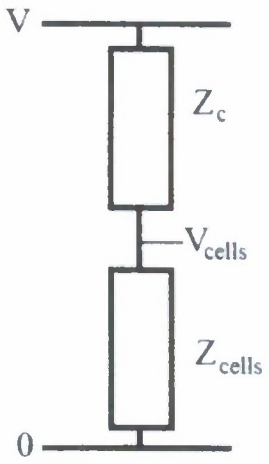

Figure 5. Equivalent circuit model which captures the voltage drop across only the cells.

The circuit in Figure 5 comprises a simple voltage divider. The voltage across the cells can be solved for with (4).

$$V_{cells} = V\left(\frac{Z_{cells}}{Z_{cells} + Z_c}\right) \tag{4}$$

Simulations

Simulations of this system were performed in MAGIC 3D. The simulation geometry is a parallel plate transmission line with the test tube containing the cell suspension in direct connection to the plates, or inserted through a hole in the transmission line plates. In the case of direct connection the voltage across the cell suspension is very similar to the applied pulse. The small capacitance across the cell suspension will add a component of displacement current that is proportional to the derivative of the voltage pulse. This effect is very small for the case of conductive connection. The voltage across the capacitively coupled case is quite different. The current that is applied to the cell suspension must be driven by displacement current from the transmission line through the test tube. The voltage across the cell suspension will have a much larger component proportional to the derivative of the transmission line voltage.

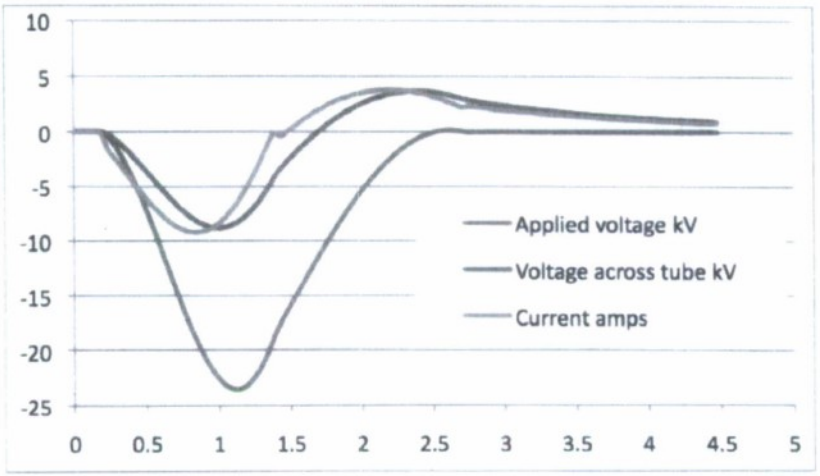

Figure 6. MAGIC simulation of capacitively coupled test tube

The output of the MAGIC simulations for the case of capacitive coupling is shown in Figure 6. The voltage across the tube, measured across the anode-cathode gap, is approximately 8 kV for a transmission line voltage of 24 kV. The value of approximately 30% of the applied voltage is consistent with our simple analytic circuit model. The electric field as seen by a cell in the center of the test tube will be V/d. Therefore, in order to apply the same electric field to cells in the conductive connection case, the electrode gap was set to be ~3 times larger than the anode-cathode gap in the capacitive coupling case.

Cell culture and imaging

Human Jurkat T cells were grown in RPMI 1640 media (InVitrogen) supplemented with penicillin, streptomycin and 10% fetal calf serum. For electroporation, cells in culture media were mixed in the absence or presence of bleomycin (5 ng/ml) and agarose (1/10 volume of 4%agarose in phosphate-buffered saline). The agarose solution was added to the glass tubes and allowed to gel at room temperature for 10 minutes. Following electroporation, the gel tubes were extruded using positive air pressure and incubated in culture media containing 5 ng/ml DAPI. Cells were imaged 2 hours after electroporation to determine immediate killing and 24 hours after death to determine death due to the bleomycin..

Experimental Results

The experiments were performed in order to determine what effect the bipolar, capacitively coupled pulses have on the cells.

In order to minimize movement of the cells within the tubular glass electroporation chamber, the Jurkat cells were embedded in an agarose solution which was then allowed to solidify. The cells remained viable by both trypan blue staining assays and growth assays when cultured within the gel matrix. The cells within the electroporation chambers were subjected to electroporation with either direct connection or with capacitance coupling in both the presence and absence of bleomycin, a cancer chemotherapeutic drug which has been used previously in experiments to determine electrochemotherapeutic effects on Jurkat cells. Following electroporation, cells were stained by culturing in growth medium containing DAPI. DAPI does not stain cells with an intact cellular membrane, but if membrane integrity is compromised cell nuclei will be stained. Each of the agarose tube gels containing Jurkat cells was stained and counted (Figure 7) for dead (DAPI positive) and live (DAPI negative) cells.

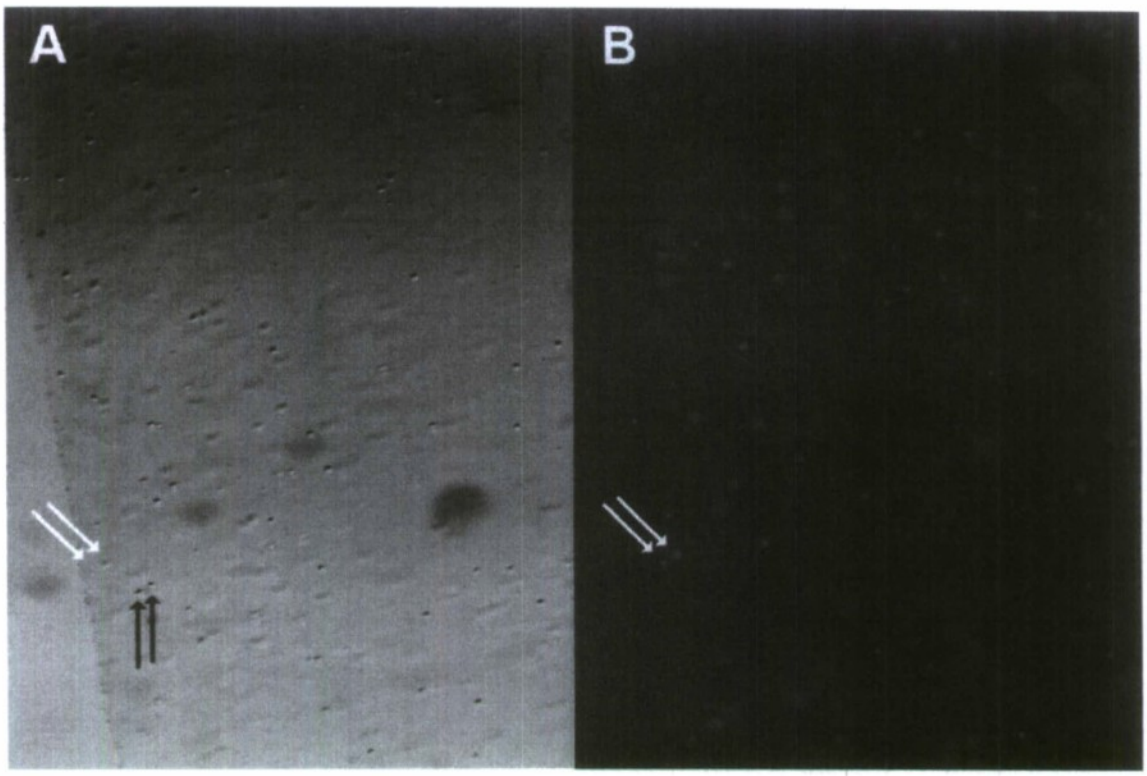

Figure 7. DAPI Staining of Jurkat Cells Immobilized in Agarose. Panel (A) shows a bright field image of an agarose gel containing immobilized Jurkat cells. The edge of the gel is evident along the lower left image of the gel. Two dead cells that are stained with DAPI are indicated with white arrows and two live cells that are not stained with DAPI are indicated with black arrows. Panel (B) shows the corresponding fluorescence image and the two dead cells indicated in panel A are also indicated with white arrows in panel B.

Jurkat cells immobilized in gels with our without bleomycin were subjected to either capacitive coupling or direct current. Cells received 0, 1, 4 or 8 pulses under each of these conditions and then cell survival was quantitated as described in Figure 7 for cell survival. Three replicate tube gels were quantitated for each experimental point. At two hours following electrical treatment (Figure 8) a significant difference in cell survival was seen for those cells that underwent conductive current treatments. After 8 pulses, the cells treated with conductive

current were reduced to less than 40 % of their starting population. The cells that received capacitive coupling were not significantly reduced in number after 2 hours.

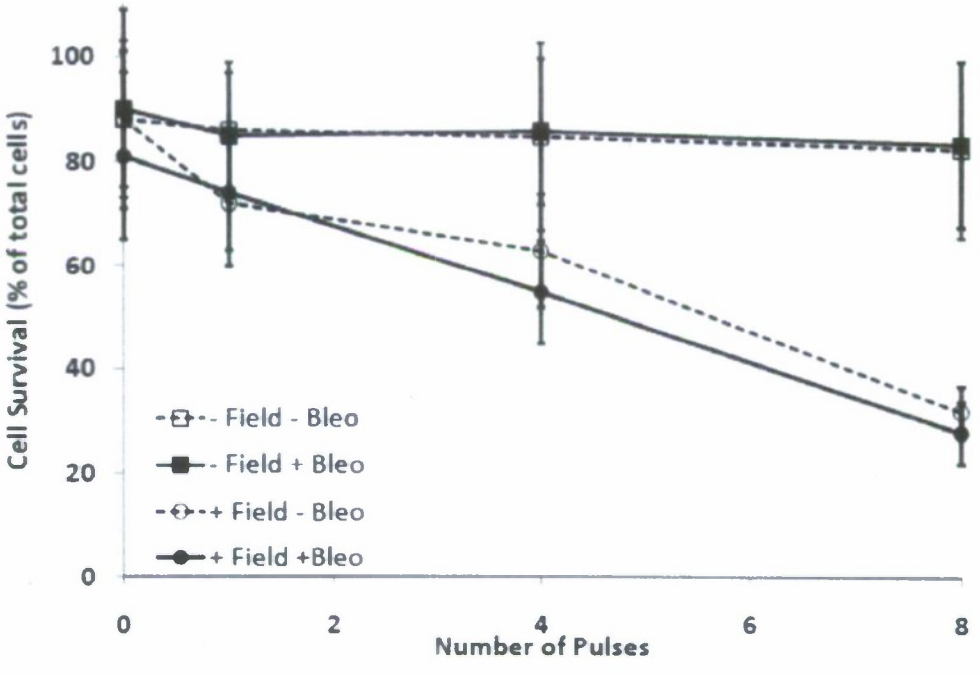

Figure 8. Cell Survival at 2 Hours Following Electroporation. Jurkat cells were pulsed in the absence (-Bleo) or presence (+Bleo) with either capacitive current (-Field) or direct coupling (+Field) for the indicated number of pulses. Two hours after treatment, cells were incubated in the presence of DAPI and cell survival determined.

In order to measure the electrochemotherapeutic effect of treatments, cells were incubated in the agarose tubes for 24 hours and then imaged for DAPI staining. As shown in Figure 9, the cells treated with direct connection showed significantly lower survival after 24 hours compared to that seen at 2 hours (Figure 8). In the presence of bleomycin and following direct coupling, cell survival as reduced to 10% after 4 pulses. In the absence of bleomycin, cell survival was 32%. The difference between these two survival rates is consistent with electroporation of bleomycin leading

to enhanced cell death over the 24 hour period. Similar results have been reported previously for Jurkat cells maintained in solution [13]. However, no electrochemotherapeutic effect was observed for the cells that experience capacitive coupling.

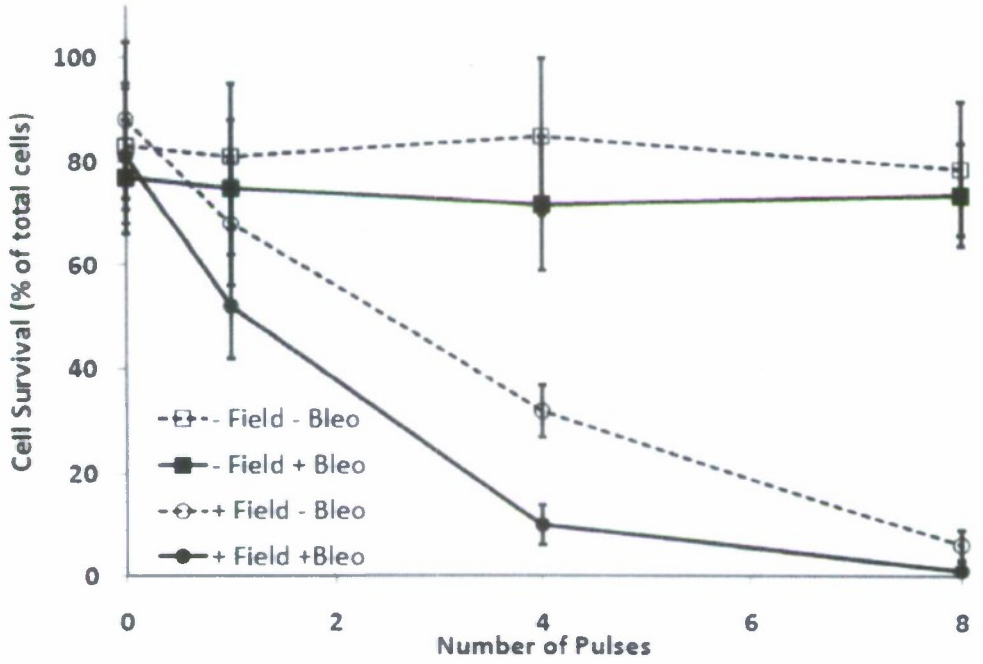

Figure 9. Cell Survival at 24 Hours Following Electroporation

Discussion

The difference between these two cases is immediately obvious. The magnitude of the electric field was 15 kV/cm in both cases, however in the case of direct-conductive connection a cell in the center of the tube will experience an electric field in one direction only, whereas a cell in the center of the tube in the capacitive coupling case will experience an electric field that reverses direction. From the simulation data of the capacitively coupled case one observes that the bipolar pulse can be integrated and the result is zero after the pulse has passed, Figure 10. The directly connected conductive case is different in that it is left with a net polarization after the pulse is applied. Previous work [2] has indicated that effects can be described a scaling law related to $E^2 \tau^2 n$, where E is the electric field τ is the pulse duration and n is the number of pulses. While this

scaling law may hold for a monopolar pulse, bipolar pulses occurring faster than the timescale of pore formation that result from capacitive coupling to the cell suspension may not be accurately described by this law since the electric field reverses and this is lost in the E^2 term.

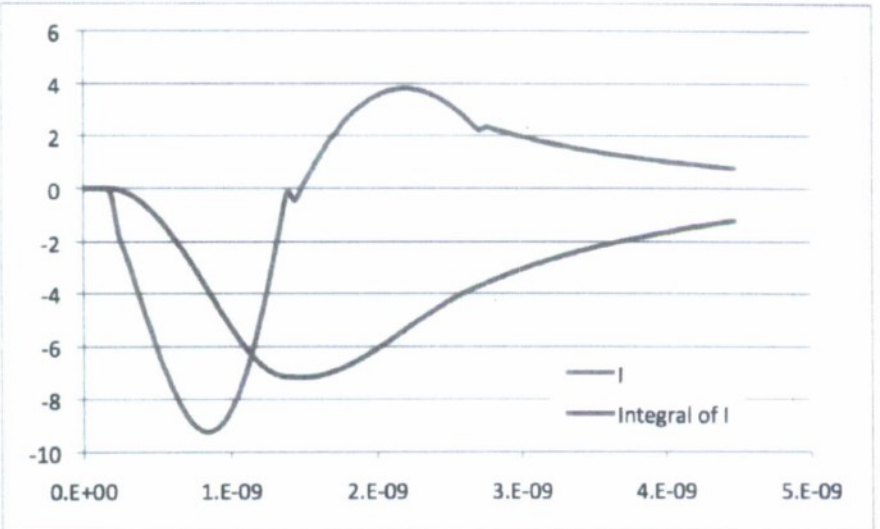

Figure 10. Simulation of capacitively coupled case showing the current through the cell suspension and the integral of the current.

References

[1] W. Rogers, J. Merritt, J. Comeaux, C. Kuhnel, D. Moreland, D. Teltschik, J. Lucas, and M. Murphy, "Strength-duration curve for an electrically excitable tissue extended down to near 1 nanosecond," *IEEE TRANSACTIONS ON PLASMA SCIENCE*, vol. 32, Aug. 2004, pp. 1587-1599.

[2] K. Schoenbach, B. Hargrave, R. Joshi, J. Kolb, R. Nuccitelli, C. Osgood, A. Pakhomov, M. Stacey, R. Swanson, J. White, S. Xiao, J. Zhang, S. Beebe, P. Blackmore, and E. Buescher, "Bioelectric effects of intense nanosecond pulses," *IEEE TRANSACTIONS ON DIELECTRICS AND ELECTRICAL INSULATION*, vol. 14, Oct. 2007, pp. 1088-1109.

[3] S. Beebe, J. White, P. Blackmore, Y. Deng, K. Somers, and K. Schoenbach, "Diverse effects of nanosecond pulsed electric fields on cells and tissues," *DNA AND CELL BIOLOGY*, vol. 22, Dec. 2003, pp. 785-796.

[4] C. Yao, Y. Mi, X. Hu, C. Li, C. Sun, J. Tang, and X. Wu, "Experiment and Mechanism Research of SKOV3 Cancer Cell Apoptosis Induced by Nanosecond Pulsed Electric Field," *2008 30TH ANNUAL INTERNATIONAL CONFERENCE OF THE IEEE ENGINEERING IN*, 2008, pp. 1044-1047.

[5] A. Pakhomov, J. Kolb, J. White, R. Joshi, S. Xiao, and K. Schoenbach, "Long-lasting plasma membrane permeabilization in mammalian cells by nanosecond pulsed electric field (nsPEF)," *BIOELECTROMAGNETICS*, vol. 28, Dec. 2007, pp. 655-663.

[6] R. Sundararajan, "Nanosecond Electroporation: Another Look," *MOLECULAR BIOTECHNOLOGY*, vol. 41, Jan. 2009, pp. 69-82.

[7] S. Beebe, P. Fox, L. Rec, K. Somers, R. Stark, and K. Schoenbach, "Nanosecond pulsed electric field (nsPEF) effects on cells and tissues: Apoptosis induction and tumor growth inhibition," *IEEE TRANSACTIONS ON PLASMA SCIENCE*, vol. 30, Feb. 2002, pp. 286-292.

[8] S. Beebe, P. Fox, L. Rec, L. Willis, and K. Schoenbach, "Nanosecond, high-intensity pulsed electric fields induce apoptosis in human cells," *FASEB JOURNAL*, vol. 17, Jun. 2003, pp. 1493-+.

[9] B. Ibey, S. Xiao, K. Schoenbach, M. Murphy, and A. Pakhomov, "Plasma Membrane Permeabilization by 60-and 600-ns Electric Pulses Is Determined by the Absorbed Dose," *BIOELECTROMAGNETICS*, vol. 30, Feb. 2009, pp. 92-99.

[10] J. Deng, K. Schoenbach, E. Buescher, P. Hair, P. Fox, and S. Beebe, "The effects of intense submicrosecond electrical pulses on cells," *BIOPHYSICAL JOURNAL*, vol. 84, Apr. 2003, pp. 2709-2714.

[11] K. Schoenbach, S. Katsuki, H. Akiyama, T. Heeren, J. Kolb, S. Xiao, T. Camp, R. Joshi, C. Osgood, R. Nuccitell, and S. Beebe, "Biological effects of intense subnanosecond electrical pulses," *Proceedings of the 27th International Power Modulator Symposium and*, 2006, pp. 573-576.

[12] K. Schoenbach, Shu Xiao, R. Joshi, J. Camp, T. Heeren, J. Kolb, and S. Beebe, "The Effect of Intense Subnanosecond Electrical Pulses on Biological Cells," *Plasma Science, IEEE Transactions on*, vol. 36, 2008, pp. 414-422.

[13] D. Jordan, M. Uhler, R. Gilgenbach, and Y. Lau, "Enhancement of cancer chemotherapy in vitro by intense ultrawideband electric field pulses," *JOURNAL OF APPLIED PHYSICS*, vol. 99, May. 2006.